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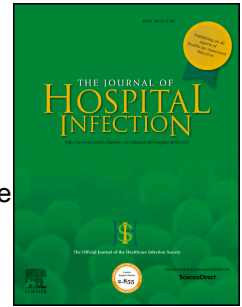
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Local outbreak of extended spectrum beta lactamase SHV2a producing *Pseudomonas aeruginosa* reveals the emergence of a new specific sub-lineage of the international ST235 high risk clone

Guilhem ROYER¹, Frederic FOURREAU¹, Boris BOULANGER², Mélanie MERCIER DARTY³, David DUCELLIER¹, Florence CIZEAU¹, Anaïs POTRON⁴, Isabelle PODGLAJEN⁵, Nicolas MONGARDON^{2,6}, Jean-Winoc DECOUSSER^{1,7}

1: Department of Bacteriology and Infection control, University Hospital Henri Mondor, Assistance Publique-Hôpitaux de Paris, Créteil, FRANCE

2: Service d'anesthésie-réanimation chirurgicale, unité d'anesthésie-réanimation chirurgicale cardio-vasculaire, DHU A-TVb, University Hospital Henri Mondor, Assistance Publique-Hôpitaux de Paris, Créteil, FRANCE

3: Next Generation Sequencing platform, University Hospital Henri Mondor, Assistance Publique-Hôpitaux de Paris, Créteil, FRANCE

4: Centre National de Référence de la résistance aux antibiotiques, Centre Hospitalier Universitaire de Besançon, France; UMR6249 CNRS Chrono-Environnement, Université de Franche-Comté, Besançon, France

5 : Department of Bacteriology, Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, Paris, FRANCE

6: Inserm, U955, Equipe 3 "Stratégies pharmacologiques et thérapeutiques expérimentales des insuffisances cardiaques et coronaires", Institut Mondor de Recherche Biomédicale, University Hospital Henri Mondor, Assistance Publique-Hôpitaux de Paris, Créteil, FRANCE

7 : EA 7380 Dynamyc Université Paris-Est Créteil (UPEC), Ecole nationale vétérinaire d'Alfort (EnvA), Faculté de Médecine de Créteil, 8 rue du Général Sarrail, 94010 Créteil, FRANCE

*Corresponding author: Dr Jean-Winoc Decousser, Department of Microbiology, Assistance Publique –Hôpitaux de Paris, University Hospital Henri Mondor, 94000 Créteil, France

Tel : 33 1 49 81 49 36, Fax : 33 1 49 81 28 39, Mail : jean-winoc.decousser@aphp.fr

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Abstract

Objectives

Pseudomonas aeruginosa is a major bacterial pathogen responsible for hospital-acquired infections. Although its epidemiology is considered as non-clonal, some particular international high-risk multidrug resistant clones have been recognized. From the first report of an intra-hospital outbreak due to a SHV2a producing *P. aeruginosa* strain, we described the emergence of a new ST235 specific lineage harboring this uncommon extended spectrum beta lactamase.

Methods

Between May and October 2018, four patients hospitalized in the cardiovascular intensive care unit of a French teaching hospital were infected by a multidrug resistant *P. aeruginosa* isolate. Serotype and antimicrobial susceptibility were tested; MLST, core genome MLST and resistome were determined through whole genome sequencing. A phylogenetic analysis based on single nucleotide polymorphism was performed using available ST235 genomes.

Results

The four strains were susceptible to colistin, ciprofloxacin, ceftazidime-avibactam and ceftolozane-tazobactam. *bla*_{SHV2a} was identified in each genome of this ST235 - O11 serotype cluster that showed an identical cgMLST profile (0 to 2 out of 4162 different alleles). The phylogenetic analysis of 162 ST235 genomes showed that only four other strains harbored a *bla*_{SHV2a} originating from France and USA, clustering together although being different than the outbreak strains.

Conclusions

Among the ST235 *P. aeruginosa* strains, a sub-lineage sharing a common genetic background and harbouring the *bla*_{SHV2a} extended-spectrum beta-lactamase seems to emerge from different locations, yielding secondary local outbreaks.

Abstract word count: 225

Introduction

Pseudomonas aeruginosa is a major bacterial pathogen responsible for hospital associated infections [1]. Although its epidemiology is considered as non-clonal, particular high-risk clones have been described, some of them carrying extended-spectrum beta-lactamases making them multidrug resistant (MDR) [2-6]. They belong to a limited number of genetic subgroups identified as particular Sequence Type (ST) through the Multi Locus Sequence Typing (MLST) system; this approach characterizes the genetic background of a group of strains in sequencing a limited number of housekeeping genes and in comparing their allelic profiles. The most relevant *P. aeruginosa* high-risk clones belong to ST111, ST175 and ST235 [2-6]. ST235 is the most prevalent of these clones [7, 8]. The emergence and spread of such a high-risk clone could modify dramatically and very quickly the landscape of the antimicrobial susceptibility on the scale of a region or a country; the changing epidemiology of the nosocomial isolates of *P. aeruginosa* in the Eastern European countries due to the clonal diffusion of a carbapenemase-producing VIM-2 positive ST235 *P. aeruginosa* clone is a perfect example of this threat [2]. The epidemiological success of the ST235 high-risk clone could be explained at least in part by (i) particular genetics backgrounds, (ii) specific virulence factors, and (iii) a great capacity to capture mobile genetic elements carrying resistance genes. Class B and class D beta-lactamases were predominantly identified in ST235; extended-spectrum class A beta-lactamases (ESBL) have been far less frequently described. The putative impact of ESBL production on the empiric therapeutic strategies targeting *P. aeruginosa* is significant given that ceftazidime is a first-line agent to treat *P. aeruginosa* infections.

We report for the first time an intra-hospital outbreak due to an ESBL-producing *P. aeruginosa* strain. Through a large whole genome sequence comparing approach, we hypothesized that we faced the emergence of a new sub lineage of the widespread ST235 clone harboring *bla*_{SHV2a} and sharing a genetic background.

Methods

The Henri Mondor university hospitals constitute a 2853 beds group of five teaching hospitals from the Assistance Publique – Hôpitaux de Paris network located in the southern suburb of Paris [9]. The cardiac branch includes a dedicated surgical and intensive care unit (ICU) ward offering a large panel of medical, interventional and surgical activities including heart transplantation. Between May and October 2018, four patients with a history of hospitalization in the cardiovascular ICU were infected by a multidrug resistance *P. aeruginosa* strain. Criteria for infection included the identification of the strain from a protected and/or invasive specimen and the validation of the infection by a multidisciplinary board including independent infectious diseases specialists and clinical microbiologists that meets weekly. Outbreak investigation was performed by the local infection control team. It included (1) collection of demographic, hospital stay and microbiologic data, (2) evaluation of clinical practices, and (3) an environmental survey. The audit of practices focused on hand hygiene compliance, including hand hygiene opportunities during patient care according to the “five moments for hand hygiene” and hand hygiene requirements (i.e. lack of ring, jewelry, polished and false nails). Additionally, the use of gloves and gowns, and procedures for disposal of human waste and water used for washing patients, were observed. The sink drain trap disinfection procedure was observed. The environmental survey consisted in sampling and analyzing the water of each faucet present in the ward. All the sink drain traps were sampled and cultured to detect ceftazidime and/or carbapenem resistant Gram negative bacilli, including those from the welfare spaces and the bedpan washer disinfectant. Basins used for washing patients were swabbed just before use. The ultrasound scanner (including its ultrasonic probe and computer key) were swabbed to identify a putative mobile reservoir of bacteria; the conditioning and the use of the ultrasound gel was investigated.

At the time of the third case diagnosis, respiratory (or throat if non-intubated) and rectal screening of all the patients present was implemented; rectal screening was undertaken at admission, and weekly thereafter. We also retrospectively screened the 2017-2018 database of the laboratory to identify additional cases.

Antimicrobial susceptibility of the strains was tested using disc diffusion method and microdilution MIC determination, accord to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2018 guidelines and interpretation criteria (http://www.eucast.org/ast_of_bacteria/). The list of antimicrobial compounds was ticarcillin, ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam, ceftazidime, cefepim, aztreonam, ceftazidime – avibactam, ceftolozane – tazobactam, meropenem, imipenem, amikacin, gentamicin, tobramycin, levofloxacin, ciprofloxacin and colistin. Serotyping was carried out at the French National Reference Center for *P. aeruginosa* (Besançon, France). Individual informed consent was obtained from each patient.

Whole genome sequences of the four strains were obtained and analyzed as previously described [9]. Briefly, whole genome sequences were obtained from Illumina NextSeq 2x150 bp and analyzed with an in-house bioinformatics pipeline. MLST typing and resistome determination was obtained from the Center for Genomic Epidemiology website (<http://www.genomicepidemiology.org>). The comparison of epidemic strains was carried out through the core genome MLST analysis (Ridom SeqSphere +, Ridom GmbH, Muenster, Germany) [10]. We search for integron using integronFinder [11] and we annotated our epidemic strains using Prokka (v1.13) with standard parameters [12]. A phylogenetic analysis through single nucleotide polymorphism (SNPs) identification was performed using additional local and public available complete genomes (RefSeq database) belonging to the same ST. The phylogenetic history of the strains was reconstructed using SNPs of the core genome: SNP calling was performed for each strain against NCGM 1900 (GCF_000829275.1) with Snippy (v4.2.3) (<https://github.com/tseemann/snippy>). Then, only core SNPs were conserved and used to construct a maximum likelihood phylogenetic tree using FastTree (v2.1.8) with general time reversible evolution model and a gamma distribution of rates across sites. All the new genomic data are publicly available through the Bioproject PRJEB32170.

Results

The stay duration, time of infection and location of the cases hospitalized in the Cardiac Surgical Ward and Intensive Care Unit is reported in the Figure 1. The four isolates showed a MDR phenotype associated to a positive double-disk synergy test between cefepime and ticarcillin-clavulanic acid; they were only susceptible to colistin (MIC= 2 mg/L), ciprofloxacin (MIC= 2 mg/L), ceftazidime-avibactam (MIC= 4 mg/L), and ceftolozane-tazobactam (MIC= 2 mg/L). The ceftazidime MIC varied according to the origin of the stains, from 8 mg/L to 32 mg/L. These strains were responsible for severe infections (ventilated-associated pneumonia, mediastinitis, osteitis) in patients hospitalized since at least 3 weeks and having received several courses of broad-spectrum antibiotic therapy (Figure 1, Table I). None of the four patients had been hospitalized or had travelled abroad during the previous twelve months, including the last case-patient who was a Serbian origin woman.

Infection control investigation highlighted gaps in hand hygiene compliance. 272 hand hygiene opportunities by 42 healthcare professionals (doctors: 8; nurses: 19, caregivers: 10, others including physiotherapists and students: 5) were observed. 10% of hand disinfections were considered as non-conforming with hand hygiene requirements. Of the remaining 244 opportunities, overall observance was 56% (before touching a patient: 46%, before clean/aseptic procedures: 35%, after body fluid exposure/risk: 70%, after touching a patient: 62%; after touching patient surroundings: 62%). Regarding the 39 uses of gloves, only 25 (64%) were relevant and a hand hygiene was performed before and after this use for only 17 (43%) of the observed moments. Staff wore the correct protective attire for disposal of human waste and water used for bathing patients. However, wash water was disposed of into the washbasin in the patient's room instead of the bedpan washer disinfectant. There was no formal system for disinfection of sink traps.

The environmental survey highlighted contamination of sink drain traps, whereas the water sample of the eight faucets was free of *P. aeruginosa* and enterobacteria, excepted for the water collected from a showerhead in a technical room. By contrast, 8 out of 15 sink drain traps contained ESBL-producing enterobacteria and/or multidrug-resistant *P. aeruginosa*. Moreover, an NDM-producing *Klebsiella pneumoniae* was isolated from one sink drain trap. None of the *P. aeruginosa* strains isolated from the environment showed an ESBL

phenotype. Some wild type *P. aeruginosa* strains were identified from the basins used for the body wash and swabbed just before their use. These plastic basins were macroscopically altered. No relevant bacteria were isolated from the ultrasound scanner.

As results of these findings, the infection control team promoted hand hygiene compliance and use of the bedpan washer disinfectant to discard putatively contaminated effluents. Sink drain taps were disinfected with bleach every week. The altered plastic basins, and the contaminated showerhead were discarded. During the outbreak, all patients were screened weekly for MDR organism carriage. No additional cases were identified prospectively or retrospectively. A strict antimicrobial stewardship policy was maintained including the continuation of a weekly formalized meeting with infectious diseases specialists and daily interactions.

Regarding the whole genome sequencing, the number of contigs varied between 62 and 118 for the four strains; their size and GC% were identical (66.0% and 6.630 kbp, respectively). A *bla*_{SHV2a} gene was identified in each genome that confirmed the ESBL phenotype. The other acquired resistance genes as well as the mutations in GyrA, ParC, AmpD and OprD were also common to the four isolates (Table S1). The MLST and serotyping identified a ST235 - O11 cluster of strains. As previously reported for all the ST235 genomes, the *exoU* gene encoding for a Type III secretion system exotoxin was present in the four genomes [8]. The cg MLST ad hoc scheme was constructed from a pool of 4162 genes; 98.3% of the core genome genes were identified in all the analyzed sequences. The four strains showing an identical cgMLST profile (0 to 2 out of 4162 different alleles).

Phylogenetic analysis of the 162 ST235 genomes (including 155 genomes from the RefSeq database, three genomes from the local database and the four studied strains) showed that four additional strains harbored a *bla*_{SHV2a}; they originated from France (n=2) and USA (n=2) and clustered together, although being different than our 4 outbreak isolates (Table I, Figure 2, Table S2). The two French strains were isolated and sequenced during an outbreak of *bla*_{VIM-1} *P. aeruginosa* in another hospital from a different town (Hôpital Européen Georges Pompidou, Paris, France); the corresponding patients had no relation to or contact with our hospital. The origin of the two American genomes were human and the hospital setting without other details. Their resistome was reported in Table S1. All the eight strains

harbored a class 1 integron. The genetic location of the *bla*_{SHV-2a} cannot be determined due to the shortness of the contigs carrying it. However, genomic context analysis revealed in three out of four strains from our hospital the same organization as the one observed by Hammami et al. from Tunisian *bla*_{SHV2a} *P. aeruginosa* isolates; unfortunately, in this report the ST of the isolates was not performed [13]. In our four strains the *bla*_{SHV-2a} gene seems to be mobilized through IS26 insertion sequence. Moreover, this sequence has integrated into a class 1 integron (Figure 3).

Discussion

Through an outbreak of infections due to an unusual SHV2a ESBL producing *P. aeruginosa* strain, we highlighted the putative emergence in two different countries of a shared ST235 genetic background that seems to be associated with this resistance trait. Among the several international high-risk clones that have spread worldwide, the ST235 is the most successful one [6,8]. Among the class A beta-lactamases identified in this high risk clone, *bla*_{GES}, *bla*_{PER} and *bla*_{KPC} predominated; the *bla*_{SHV2a} was not previously reported [6].

The description of new sublineage of high-risk clones combining a successful genomic background and a particular acquired resistance gene armamentarium is of interest [14]. From a public health point-of-view, the local selection and emergence of a MDR sublineage could lead to its internationally spread. Thus, the ST235 widespread clone seems to have emerged in France thirty years ago [8]. The epidemiology of resistant *P. aeruginosa* in France is quite reassuring [15]. Nevertheless, epidemic high-risk clones are still present in French ICUs, including ST235 [16]. Therefore, the spread of MDR sublineage belonging to the ST235 genetic background must be carefully monitored. Secondly the worldwide spread of numerous MDR bacteria and the limited resources in term of infection control measures (dedicated health care workers, single rooms, consumables as gloves and gowns) lead us to focus on the most virulent clones or those with the most important capacity to spread in and out the hospital environment. From an infection control approach, the management of a MDR *P. aeruginosa* strain from a patient or an environmental sampling is still debated: European guidelines support the implementation of contact precautions in an epidemic setting but with a low level of evidence [17]. Our work supports the implementation of strict

hand hygiene and contact precautions as soon as a multi-drug resistant *P. aeruginosa* belonging to a high clone is identified, at least in a high risk environment as an ICU. The identification of a high risk clone could be a relevant approach to trigger appropriately costly infection control measures. Rapid and dedicated laboratory detection method must be developed to identify in real time these strains requiring reinforced infection control measures. Bench-top whole genome sequencer or MALDI-TOF adapted technology could be of interest [18]. Regarding the infection control investigation, the cross transmission pathway from patient-to-patient through healthcare contacts is clearly the main hypothesis, supported by the overlapping stays of the four patients. The role of the last diagnosed case could be debated: this case could be the index case, unidentified during his stay in the ICU and in the surgical ward at the beginning of 2018. This hypothesis is unlikely because numerous screening and diagnostic tests were performed during his stay without identifying the epidemic strain. The second option was a cross transmission between this patient and the first diagnosed one during their stay in the surgical ward or through a secondary environmental reservoir in the ICU. We failed to identify an environmental reservoir that could explained at least in part the onset of the outbreak, as reported for other published episodes of multidrug resistant *P. aeruginosa* outbreak [19]. Nevertheless we identified some gaps in infection control standards, such as hand hygiene, misuse of the patient sinks to discard wash water, and the lack of scheduled sink disinfection. It is well established that bathing practices, and especially the management of basins, constitute a potential source of bacteria transmission [20].

Finally, from a microbiological point a view the successful association of a genomic background and a particular acquired resistance gene raised the issue of the corresponding genetic and phenotypic support of this synergistic effect [7, 14]. The success of the ST235 clone was at least in part linked to the presence of specific genes promoting foreign DNA capture, leading to insertion of genetic element as transposon, integron or genomic islands harboring resistance genes; the absence of CRISPR/CAS system reinforced this capacity [7, 8]. Additionally, virulence factors as type III secretion system or elastase could play a significant role [21]. In parallel, the host genetic factors could interact with the bacterial characteristics to modulate the severity of infection [21]. The description of a new successful

combination of genetic background and resistance gene constitutes a new opportunity for further studies.

Our work has several limitations. First the number of genomes included in the phylogenetic analysis is limited, originating from two countries. This is due at least in part to the low prevalence of SHV2a producing *P. aeruginosa* among available genomes of ST235 *P. aeruginosa*. Secondly, we only reported and described the genetic content of these “subclone”, focusing on the presence/absence of genes. This first report must be completed by additional experiments, as genes expression studies, fitness and virulence testing in an infection model [22]. The experiments could objectivize an inner higher transmission potency that was not proved from the reported outbreak: the infection control investigation highlighted a lack of basic hygiene that could have promoted the spread of whatever multidrug resistant strain.

In conclusion, the report of this new putatively successful sublineage and the future fine description of the relationship between genes from diverse functions constitute a further step and opportunity to understand the genomic microbial epidemiology of *P. aeruginosa*. Moreover, the emergence of this high-risk clone must be thoroughly monitored, owing to the significant threat associated to its spread at a local, regional or nationwide scale.

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Conflict of interest statement

Authors declare no competing interests.

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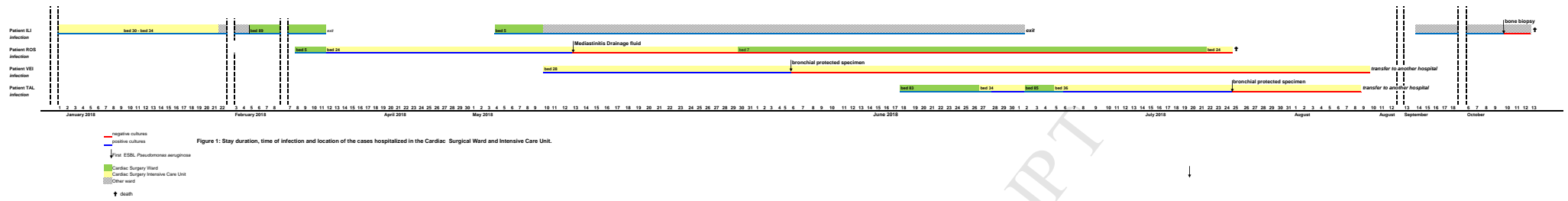
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1 **Table I: List and characteristics of the ST235 *Pseudomonas aeruginosa* strains harboring a *bla*_{SHV2a}.** Using the TAL strain as a reference, the
 2 number of SNPs from coding and non-coding regions was reported.

3

Name of the strains	Location	Year of isolation	Origin (site of isolation)	Associated bacteria	Treatment	Outcome	Number of SNPs/Indel	Genome Identification
TAL	Créteil, France	2018	Infection (ventilator associated pneumonia)	none	Ceftazidime + amikacine then ceftazidime – avibactam	Successful treatment	0	This study
ILI	Créteil, France	2018	Infection (osteitis)	<i>Enterobacter aerogenes</i> +methicillin resistant <i>Staphylococcus aureus</i>	Ceftolozane tazobactam+ vancomycin	Fatal outcome	7	This study
ROS	Créteil, France	2018	Infection (mediastinitis)	<i>Morganella morganii</i> + ESBL producing <i>Enterobacter cloacae</i> and <i>Klebsiella pneumonia</i> +methicillin resistant <i>Staphylococcus</i>	Meropenem + colistin + clindamycin then ceftazidime – avibactam + clindamycin	Successful treatment but final fatal outcome	7	This study

				<i>epidermidis</i>				
VEI	Créteil, France	2018	Infection (ventilator associated pneumonia)	none	Colistin then Ceftolozane - tazobactam	Successful treatment	3	This study
MAL	Paris, France	2018	Carriage	Unknown	No treatment	Unknown	644	Local database
KOT	Paris, France	2018	Carriage	Unknown	No treatment	Unknown	1984	Local database
GCF_001921065	USA	2012	Unknown	Unknown	Unknown	Unknown	1530	Ref Seq
GCF_001452305	USA	2005	Unknown	Unknown	Unknown	Unknown	945	Ref Seq



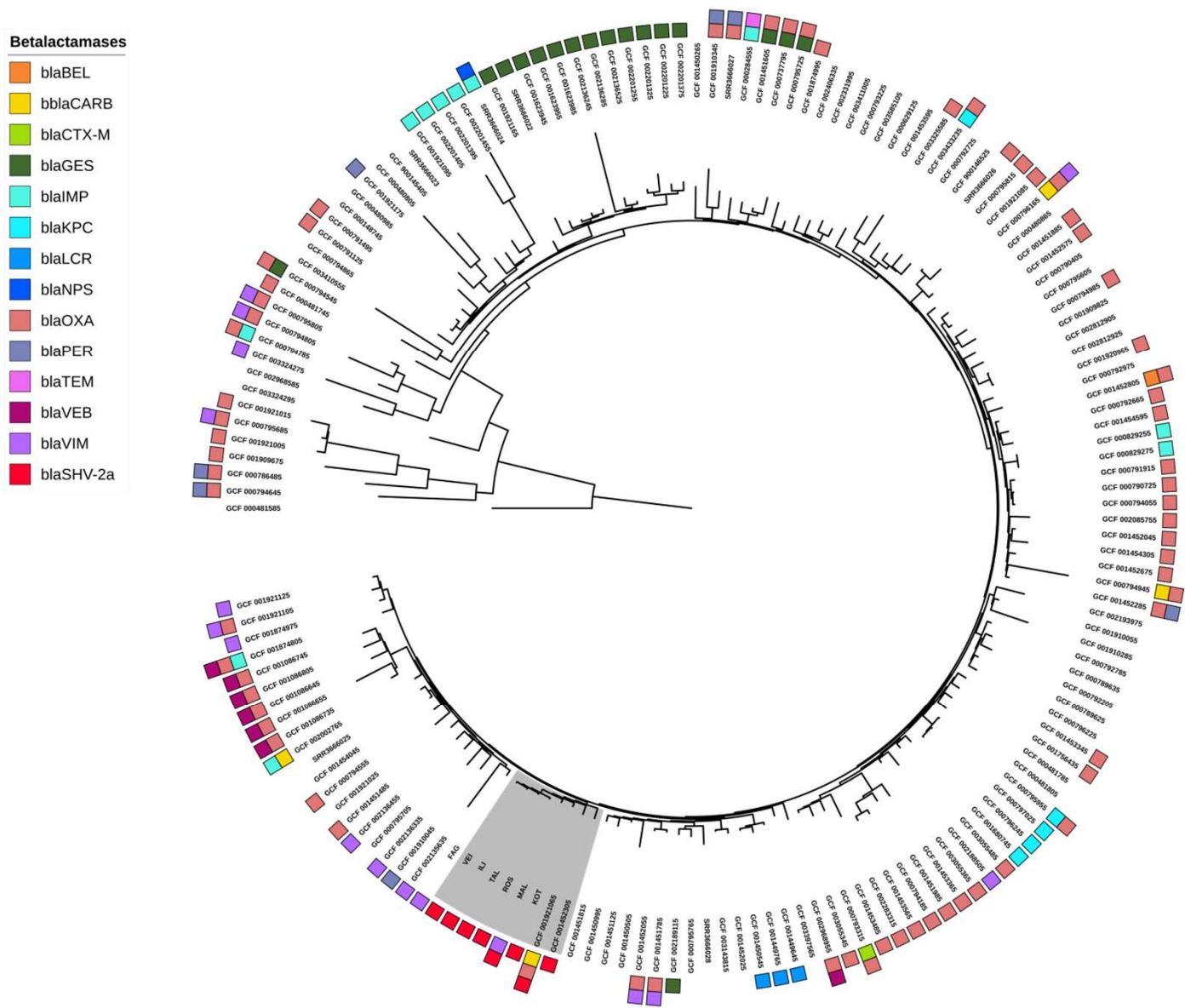


Figure 2: Maximum likelihood tree of the 162 ST235 *P. aeruginosa* reconstructed from 6944 core SNPs. The presence of acquired betalactamases coding gene is indicated outside the strain ID circle according to the given code. Naturally encoded betalactamases (*bla*_{OXA-50} and *bla*_{PAO}) were excluded. The ST235 *bla*_{SHV-2a} carrying strains are highlighted in grey. The list of betalactamases identified in each ST235 isolate was reported in Table S1 and S2.

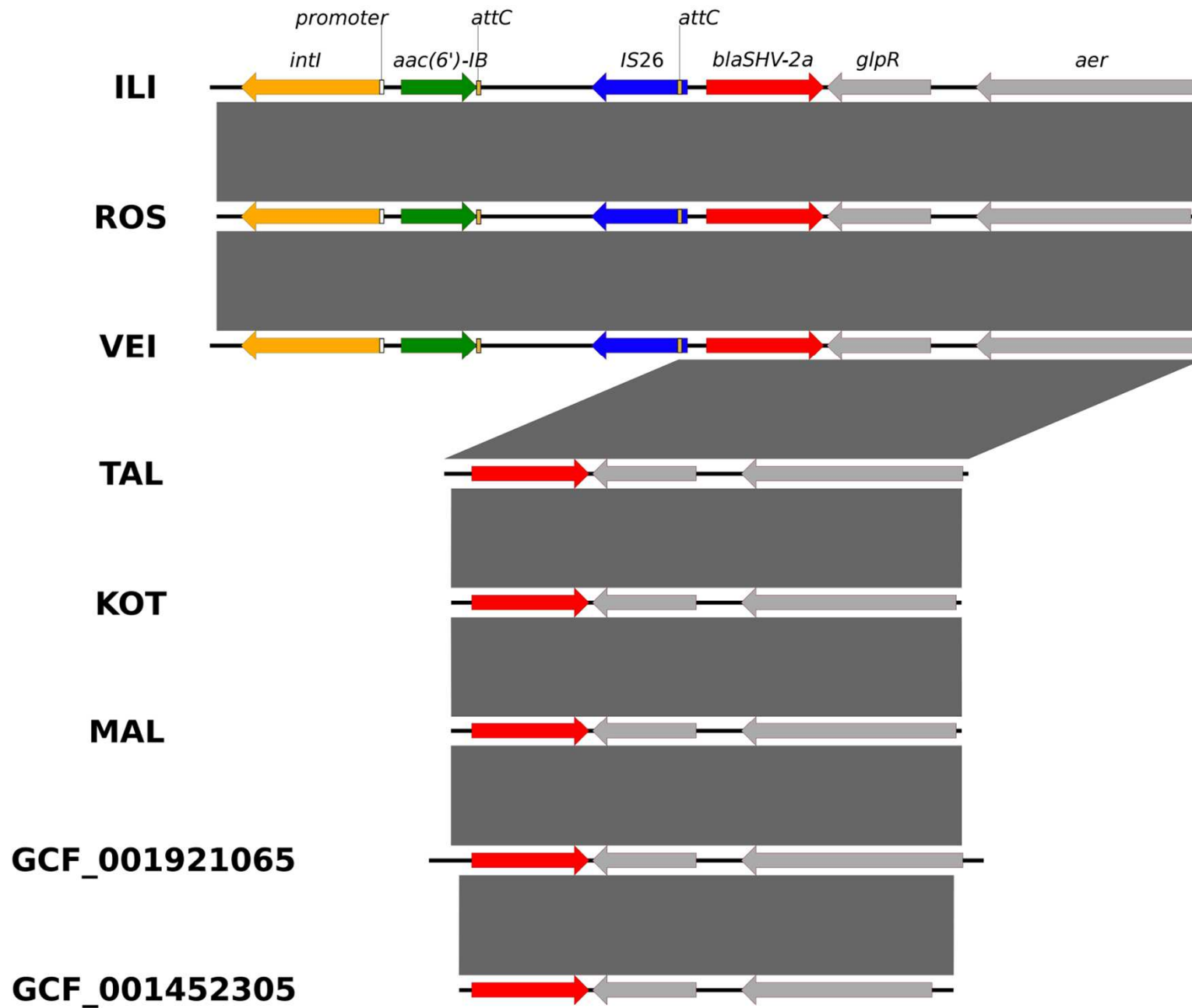


Figure 3 : Genomic context of *blaSHV-2a* strains. BlastN identical sequences are joined by grey zone.